MOLECULAR ENZYMOLOGY OF THE GLYOXALASE SYSTEM

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SUMMARY

The glyoxalase system catalyzes the conversion of 2-oxoaldehydes into the corresponding 2-hydroxyacids. This biotransformation involves two separate enzymes, glyoxalase I and glyoxalase II, which bring about two consecutive reactions involving the thiol-containing tripeptide glutathione as a cofactor. The physiologically most important substrate methylglyoxal is converted by glyoxalase I into *S*-D-lactoyl-glutathione in the first reaction. Subsequently, glyoxalase II catalyzes the hydrolysis of this thiolester into D-lactic acid and free glutathione. The structures of both enzymes have been obtained via molecular cloning, heterologous expression, and X-ray diffraction analysis. Glyoxalase I and glyoxalase II are metalloenzymes and zinc plays an essential role in their diverse catalytic mechanisms. Both enzymes appear linked to a variety of pathological conditions, but further investigations are required to clarify the different physiological aspects of the glyoxalase system.

KEY WORDS

glutathione, glyoxalase I, glyoxalase II, thiolester, methylglyoxal, zinc, enzyme evolution, catalytic mechanism, stereoselectivity, mitochondrial glyoxalase II, glyoxalase and disease, promine-retine hypothesis

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This review is a tribute to Albert Szent-Györgyi, whom I have encountered as a vibrant speaker and engaging author /1/. He is remembered as an inspiring scientist with a humorous spirit. In the vein of Szent-Györgyi's presentations, this survey is given from my personal perspective. Several reviews covering additional aspects of the glyoxalase system are available /2-6/.

THE CHEMISTRY OF THE GLYOXALASE REACTION

In 1913 it was discovered that biological systems could convert methylglyoxal into lactic acid and phenylglyoxal into mandelic acid /7-9/. In the case of methylglyoxal the possibility that the substrate could be an intermediate in glycolysis was considered, but elucidation of the glycolytic pathway by Embden and Meyerhof rendered the glyoxalase reaction into oblivion. Nevertheless, methylglyoxal was later identified as an obligatory toxic byproduct of the triose phosphate isomerase reaction of glycolysis /10/, and the glyoxalase reaction was recognized to have a scavenger role and protect cells against toxicity. Phenylglyoxal is not a normal product of metabolism, but can arise from xenobiotic substrates, as could other 2-oxoaldehydes. It has been suggested that terminal epoxides, vicinal diols or amines may similarly give rise to toxic 2-oxoaldehydes that can be detoxified by conversion to corresponding 2-hydroxyacids by the glyoxalase system /11/. However, the current opinion is that the major cellular function of glyoxalase is to inactivate methylglyoxal. The flux of reactants through the glycolytic pathway determines the output of toxic methylglyoxal, and when anaerobic energy production increases the glyoxalase reaction becomes increasingly required. This is reflected in many cancer cells, which frequently have enhanced glyoxalase activity /12/, possibly as a result of a transition from aerobic to anaerobic energy metabolism. In a proteomics study of human ovarian cancer it was demonstrated that glyoxalase I is one of a small number of proteins that are uniquely overexpressed in highly invasive tumors in comparison to tumors with low malignant potential /13/. Szent-Györgyi proposed that methylglyoxal could be a natural inhibitor (retine) of cell growth and that glyoxalase therefore was a promoter of cell proliferation (promine) by catalyzing the inactivation of methylglyoxal /14,15/. Accordingly, inhibitors of glyoxalase would be expected to suppress the growth of cancer cells, and could find clinical use as anti-

cancer drugs. His simplistic hypothesis for the regulation of cell growth is outdated, but glyoxalase is still a possible drug target in tumor cells, since inhibition of glyoxalase would enhance the toxic methylglyoxal concentration, which could lead to arrest of cell growth and apoptosis /16/.

In his attempts to purify glyoxalase, Efraim Racker discovered that the transformation of methylglyoxal into lactic acid actually takes place by means of two consecutive chemical reactions catalyzed by two discrete enzymes, which he named glyoxalase I and glyoxalase II /17/. The involvement of the intracellular tripeptide glutathione as a cofactor in the glyoxalase reaction had already been established /18/, and Racker found that glyoxalase I catalyzed the formation of the thiolester *S*-lactoylglutathione from methylglyoxal and glutathione. The thiolester is subsequently hydrolyzed by glyoxalase II to yield lactic acid, and regenerate glutathione (Fig. 1).

Early indications suggested that lactic acid produced by the glyoxalase system was of the D-configuration, in contrast to the L-isomer formed by reduction of pyruvate catalyzed by lactate dehydrogenase in mammalian tissues. Ekwall and Mannervik isolated the intermediate *S*-lactoylglutathione formed by glyoxalase I and demonstrated that the lactoyl group of the glutathione thiolester was indeed the D-isomer rather than the L-enantiomer /19/. This stereoselectivity is remarkable, since the reaction catalyzed by glyoxalase I proceeds

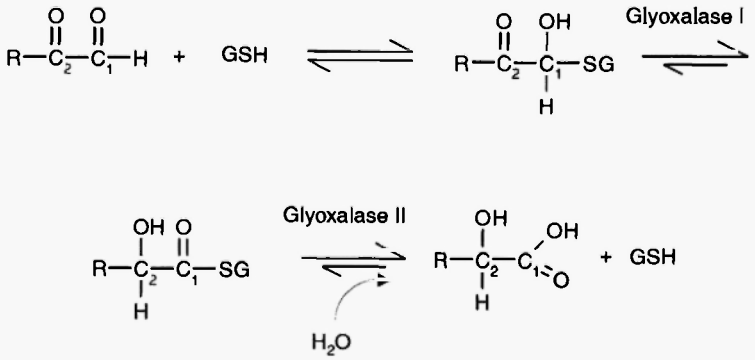

Fig. 1: The consecutive reactions catalyzed by the glyoxalase system, e.g. the conversion of methylglyoxal into D-lactic acid. GSH = glutathione.

via a racemic mixture of the hemimercaptal (also called thiohemiacetal) enantiomers formed between methylglyoxal and glutathione. The enzyme clearly has the ability to accept both enantiomers as substrates, but delivers a single chiral product of the reaction /20/. In principle, stereoselective production of D-lactic acid could have been accomplished by a non-enantioselective glyoxalase I reaction followed by a stereoselective hydrolysis of the *S*-lactoyl glutathione catalyzed by an enantioselective glyoxalase II. However, glyoxalase II does not show such substrate specificity for its thiolester substrate, and the observed stereoselective conversion of methylglyoxal into D-lactic acid is based on the stereospecificity of glyoxalase I.

The reactions catalyzed by the glyoxalase system are for all practical purposes irreversible, but the reaction catalyzed by glyoxalase I can be measured in the reverse direction from *S*-lactoylglutathione to the hemimercaptal adduct of glutathione and methylglyoxal /21/. The adduct is in equilibrium with methylglyoxal and glutathione in free form, and the reverse reaction can be driven by trapping glutathione by a chemical reaction. From the kinetic data the equilibrium constant for the isomerization of the hemimercaptal adduct and the thiolester has been calculated as $1.1 \cdot 10^4$ /21/. The methylglyoxal is predominantly hydrated in aqueous solution, but direct evidence for a role of glyoxalase I in catalyzing the dehydration and formation of the hemimercaptal adduct of the methylglyoxal could not be obtained by rapid kinetics /22/.

The glyoxalase II reaction is also essentially irreversible. The substrate *S*-lactoyl glutathione is a thiolester, with an expected negative free energy of hydrolysis ($-\Delta G$) of the order of 8 kcal/mol, similar to adenosine triphosphate. The equilibrium constant for the formation of the hemimercaptal adduct from methylglyoxal and glutathione has been estimated as $3.3 \cdot 10^2$ M^{-1} /2/, corresponding to a ΔG value of -3.5 kcal/mol. The net free energy of conversion of methylglyoxal into D-lactic acid is therefore approximately -11 kcal/mol, demonstrating the strongly exergonic nature of the chemical transformation.

ZINC IN THE ACTIVE SITES OF GLYOXALASE I AND GLYOXALASE II

The development of affinity chromatography matrices for purification of glyoxalase I /23/ and glyoxalase II /24/ facilitated detailed structural and functional characterization. Both enzymes are metallo-

proteins and appear naturally to be dependent on zinc in their active sites /25,26/. A wide range of bivalent metal ions can substitute for zinc in glyoxalase I from mammalian sources, and several of them afford enzyme activities of similar magnitude to the zinc-containing glyoxalase I /27,28/. This promiscuous metal dependence is unusual among metalloenzymes, and the possibility that glyoxalase I could act with alternative metals under physiological conditions cannot be excluded. Nevertheless, glyoxalase I is generally accepted as a zinc metalloenzyme.

CATALYTIC MECHANISM OF GLYOXALASE I

The essence of the reaction catalyzed by glyoxalase I is isomerization of a hemimercaptal into the corresponding 2-hydroxyacid. The chemical transformation proceeds via an enediol intermediate stabilized by the active site metal (Fig. 2). The reaction involves abstraction of a proton from carbon 1 and reinsertion of the proton at carbon 2. This proton transfer takes place with limited proton exchange with the surrounding medium /29,30/, and it was therefore originally assumed that a hydride transfer was involved. In the absence of substrate or product, the metal in human glyoxalase I is coordinated with two water molecules /31/ in addition to the side-chains of Gln-33 and Glu-99 in the same subunit and His-126 and Glu-172 from the neighboring subunit /32/. During the catalytic process the water molecules are displaced by the incoming substrate /31,33/. Glu-172 serves as the acid/base in the catalytic mechanism. Transition state analogs have been synthesized (Fig. 3), and the crystal structure of the complex between a transition-state analog and glyoxalase I (Fig. 4) lends support to the proposed enediol intermediate /33/.

CATALYTIC MECHANISM OF GLYOXALASE II

Glyoxalase II catalyzes a simple hydrolysis of the thiolester *S*-lactoylglutathione. Other *S*-2-hydroxyacylglutathione derivatives and structurally related substances can serve as alternative substrates /2,3/. The catalytic process involves a water molecule activated by a binuclear metal center /26/, with similarities to reactions brought about by metal-dependent β-lactamases (Fig. 5). The mechanism is distinct

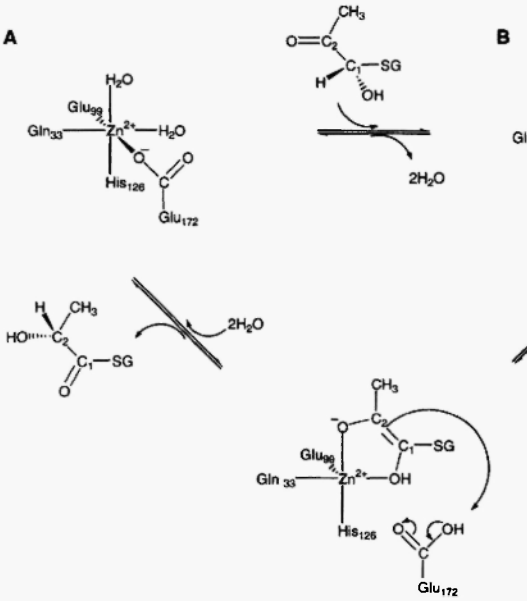

Fig. 2: Catalytic cycle of glyoxalase I. **A.** Binding of the hemimercaptal adduct of methylglyoxal and glutathione to the catalytic zinc, accompanied by displacement of two water molecules. **B.** Abstraction of the C1 proton of the zinc-bound substrate by the basic Glu-172. **C.** Re-insertion of the proton from Glu-172, followed by the release of the product *S*-D-lactoyl-glutathione and rehydration of the active site zinc.

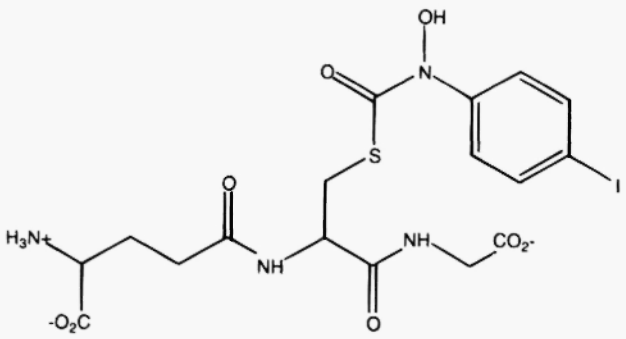

Fig. 3: Structure of the transition state analog *S*-(*N*-hydroxy-*N*-*p*-iodophenyl-carbamoyl)glutathione.

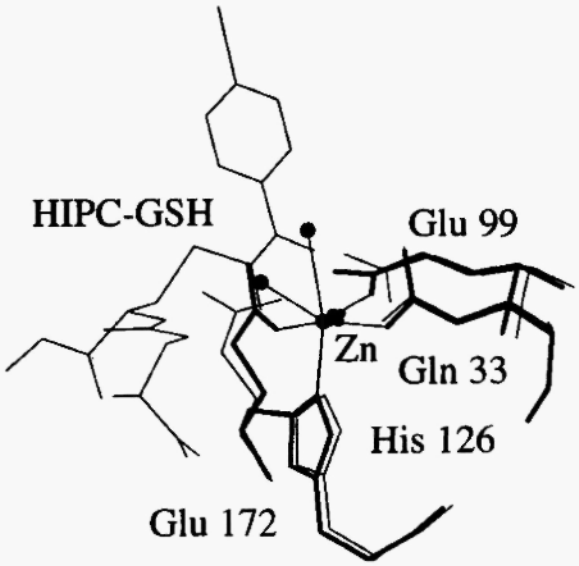

Fig. 4: Transition state analog in complex with the active site of human glyoxalase I. *S*-(*N*-hydroxy-*N*-*p*-iodophenylcarbamoyl)glutathione (HIPC-GSH) coordinated to the active site zinc ion mimics the enediolate intermediate of the catalyzed reaction. Two water molecules (filled circles above Zn) are displaced by the two oxygen atoms of the enediolate. (Figure based on PDB entry 1QIN, from /33/ with permission; © 1999 American Chemical Society.)

from that of the catalytic triad found in many hydrolytic enzymes, such as the tryptic proteases. The metal center of human glyoxalase II contains one zinc atom plus a second bivalent metal ion (Fig. 6), which could be zinc or iron /26,34/. As in the case of glyoxalase I, it is difficult to unambiguously determine the identity of the metal ions in the native protein. It cannot be excluded that there is natural heterogeneity in the metal complement of the active site.

STRUCTURAL AND EVOLUTIONARY ASPECTS

The crystal structures of human glyoxalase I /32/ and of human glyoxalase II /26/ have been determined. In spite of the fact that the product of glyoxalase I is the substrate of glyoxalase II, there are no significant similarities between the structures of the two enzymes. Neither are there any similarities to other glutathione-linked enzymes,

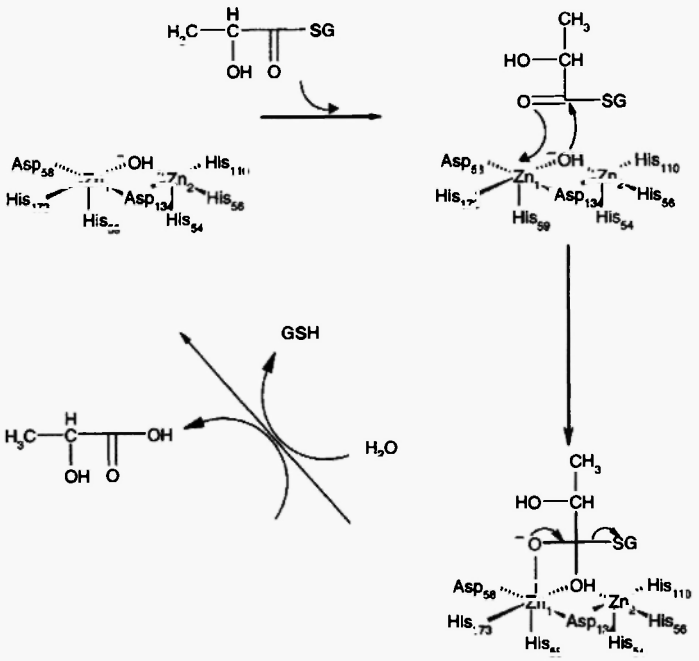

Fig. 5: Catalytic cycle of glyoxalase II. The substrate *S*-D-lactoylglutathione reacts with a hydroxyl group activated by the binuclear metal center to form a zinc-bound tetrahedral intermediate. The intermediate decomposes into free lactic acid with release of glutathione (GSH).

such as the cytosolic human glutathione transferase, glutathione peroxidase, or glutathione reductase /35/. Nevertheless, glyoxalase I has structural similarities to Fos A, a bacterial and plasmid-borne enzyme that inactivates the antibiotic fosfomycin by conjugation with glutathione /36/. Glyoxalase II has its closest structural relatives among other binuclear metal hydrolases, such as β-lactamase /37/.

The primary as well as the three-dimensional structures of glyoxalase I suggest that the evolution of the protein has involved two consecutive gene duplications. The mammalian enzyme is composed of two equal subunits, which both harbor an active site. Each of these subunits has two topologically similar domains, suggesting that they have arisen by a gene duplication /32/. In contrast, glyoxalase I from yeast has all the four corresponding domains covalently linked into a

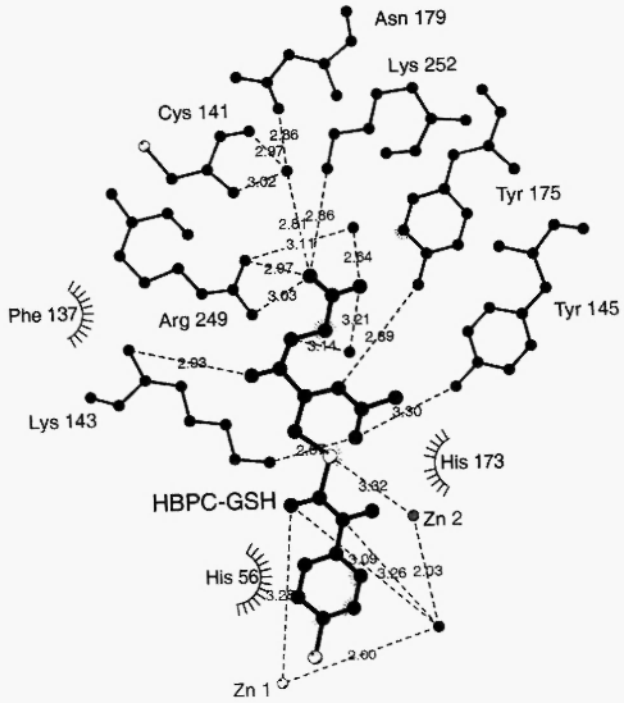

Fig. 6: Schematic structure of the active site of human glyoxalase II in complex with an active site ligand. The ligand *S*-(*N*-hydroxy-*N*-*p*-bromophenylcarbamoyl)glutathione (HBPC-GSH) is a slow substrate of the enzyme. The proposed catalytic mechanism involves a water molecule activated to a hydroxyl group by the binuclear metal center. Atomic distances are given in Ångström units. (Figure based on PDB entry 1QH5, from /26/, used with permission.)

single polypeptide chain /38,39/. In this manner the two active sites are present in the same monomer. The structure provides evidence for the proposed evolutionary gene duplications.

Glyoxalase II has a monomeric structure and its evolutionary trajectory has no obvious resemblance to that of glyoxalase I. The structural similarity of the active site region of glyoxalase II to that of the metal-dependent β-lactamases suggested that it would be possible to transform glyoxalase II into a β-lactamase by protein engineering and directed evolution (B. Mannervik, grant proposal, 2001). This has

been accomplished by extensive redesign of substrate-binding loops in combination with random mutations. The redesigned glyoxalase II has significant activity with the antibiotic cefotaxime /40/.

PHYSIOLOGICAL ASPECTS

Inspired by Szent-Györgyi's hypothesis, investigators have attempted to develop inhibitors of glyoxalase I as possible anticancer agents. Vince *et al.* /41/ have synthesized numerous glutathione derivatives and some of them are effective as glyoxalase I inhibitors at submicromolar concentrations. Among *S*-substituted glutathiones, *S-p*-bromophenylglutathione is particularly inhibitory of the human enzyme /42/. The finding that increased malignancy in solid tumors appears correlated with enhanced glyoxalase I activity /13/ suggests that glyoxalase I inhibitors may find a special role in the clinical treatment of cancer, even if the rationale is distinct from Szent-Györgyi's promine-retine hypothesis.

An important role of glyoxalase I is inactivation of methylglyoxal formed as a consequence of the hyperglycemia associated with diabetes mellitus and other diseases /4/. This important scavenger role provides protection against retinopathy and many other pathological conditions that are linked to diabetes mellitus. However, it is not clear whether any clinical procedures could be devised for upregulation of the enzyme activity and counteract methylglyoxal toxicity in patients.

A novel aspect of apparent neurophysiological significance that has attracted attention in recent years is the relationship of glyoxalase I to anxiety. Different strains of mice with divergent psychological traits show different levels of glyoxalase I activity. Some experiments suggest that anxiety and glyoxalase I activity have an inverse relationship /43,44/, whereas other studies indicate that a higher level of anxiety is positively correlated with enhanced glyoxalase I activity /45/. In the latter case, suppression of glyoxalase I by means of RNA interference was reported to have an anxiolytic effect in mice. However, behavioral studies are complex in design as well as in analysis and interpretation, and further investigations are required in order to make definitive conclusions about the association between glyoxalase I and anxiety.

Glyoxalase II has traditionally been considered as the agent through which glutathione can be regenerated from the glyoxalase I

reaction, thus completing the transformation of 2-oxoaldehydes into 2-hydroxyacids (Fig. 1). However, the enzyme is also associated with mitochondria /46,47/, suggesting that the enzyme may have other functions as well. The fact that glyoxalase II substrates are thiolesters, and therefore high-energy compounds, calls attention to the possibility that they could contribute to energy-dependent cellular processes. This aspect merits further research. Recent investigations demonstrate that cytosolic glyoxalase II, but not the mitochondrial form, protects against methylglyoxal-induced apoptosis /48/. Upregulation of the cytosolic enzyme is induced by the transcription factors p63 and p73 in the p53 family of regulatory proteins /48/. In combination with the alternative splicing that partitions glyoxalase II mRNA transcripts to become cytosolic or mitochondrial forms of the protein /47/, these observations indicate that the enzyme is involved in important functions not yet completely elucidated.

FUTURE PROSPECTS

In the approximately 95 years that the glyoxalase system has been studied, significant progress has been made in understanding the chemical transformation of the substrates and the molecular properties of the cognate biocatalysts. The protective role of glyoxalase I as an adjunct to glycolysis is well established, and can be considered as a complement to other cellular resistance mechanisms /11,49/. However, the significance of the glyoxalase system in higher and more complex physiological processes remains enigmatic. Glyoxalase I appears to be a therapeutic target in cancer cells, and studies with glyoxalase inhibitors in animal models have given promising results /50/. The biological function of glyoxalase II clearly has features in addition to the simple role of hydrolyzing thiolesters produced by the glyoxalase I reaction. Possibly, inhibition of glyoxalase II would also have therapeutic value. Gene disruption in animals or RNA interference in cultured cells may shed further light on the functions of both glyoxalase I and glyoxalase II. Novel and more incisive investigations at the cellular and organismal level are required in order to completely fathom the biological significance of the enzyme system that attracted so much of Albert Szent-Györgyi's scientific attention and ingenuity.

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